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# Development of cross-protective *Eimeria*-vectored vaccines based on apical membrane antigens

Iván Pastor-Fernández<sup>a,\*</sup>, Sungwon Kim<sup>b</sup>, Karen Billington<sup>c</sup>, Janene Bumstead<sup>c</sup>, Virginia Marugán-Hernández<sup>a</sup>, Tatiana Küster<sup>a</sup>, David J. P. Ferguson<sup>d</sup>, Lonneke Vervelde<sup>b</sup>, Damer P. Blake<sup>a</sup>, Fiona M. Tomley<sup>a</sup>

<sup>a</sup> Department of Pathobiology and Population Sciences, Royal Veterinary College, University of London, Hertfordshire, United Kingdom.

<sup>b</sup> The Roslin Institute and R(D)SVS, University of Edinburgh, Easter Bush, Midlothian, United Kingdom.

<sup>c</sup> Institute for Animal Health, Compton, Berkshire, United Kingdom.

<sup>d</sup> Nuffield Department of Clinical Laboratory Science, Oxford University, John Radcliffe Hospital, Oxford, United Kingdom.

\* Corresponding author.

E-mail address: ipastorfernandez@rvc.ac.uk

## ABSTRACT

Recently, the availability of protocols supporting genetic complementation of *Eimeria* has raised the prospect of generating transgenic parasite lines which can function as vaccine vectors, expressing and delivering heterologous proteins. Complementation with sequences encoding immunoprotective antigens from other *Eimeria* spp. offers an opportunity to reduce the complexity of species/strains in anticoccidial vaccines. Herein, we characterise and evaluate EtAMA1 and EtAMA2, two members of the apical membrane antigen (AMA) family of parasite surface proteins from *Eimeria tenella*. Both proteins are stage-regulated, and the sporozoite-specific EtAMA1 is effective at inducing partial protection against homologous challenge with *E. tenella* when used as a recombinant protein vaccine, whereas the merozoite-specific EtAMA2 is not. In order to test the ability of transgenic parasites to

confer heterologous protection, *E. tenella* parasites were complemented with *EmAMA1*, the sporozoite-specific orthologue of *EtAMA1* from *E. maxima*, coupled with different delivery signals to modify its trafficking and improve antigen exposure to the host immune system. Vaccination of chickens using these transgenic parasites conferred partial protection against *E. maxima* challenge, with levels of efficacy comparable to those obtained using recombinant protein or DNA vaccines. In the present work we provide evidence for the first known time of the ability of transgenic *Eimeria* to induce cross protection against different *Eimeria* spp. Genetically complemented *Eimeria* provide a powerful tool to streamline the complex multi-valent anticoccidial vaccine formulations that are currently available in the market by generating parasite lines expressing vaccine targets from multiple eimerian species.

## HIGHLIGHTS

- *Eimeria tenella* harbours four different stage-specific AMA1 paralogues.
- *EtAMA1*, but not *EtAMA2*, is involved in sporozoite invasion.
- *EtAMA1*, but not *EtAMA2*, induces significant protection against *E. tenella* challenge.
- Vaccination with transgenic *E. tenella* [*EmAMA1*] parasites induces partial protection against challenge with *Eimeria maxima*.

**Keywords:** *Eimeria tenella*, *Eimeria maxima*, Apical membrane antigen, AMA1, AMA2, Vaccine delivery vector, Cross protection, Heterologous protection

## 1. Introduction

Coccidiosis is caused by apicomplexan parasites of the genus *Eimeria*. Seven species replicate in chickens resulting in acute, self-limiting, enteritis that ranges in symptoms and severity depending on the species and infective dose of parasite, and the age, gender and genotype of the host. Recovered animals develop immunity that protects them against challenge with the same species of parasite, although their productivity may have been severely compromised by the disease, and repeated rounds of natural re-infection may be needed to induce full immunity (Shirley et al., 2005). The economic impact of poultry coccidiosis is estimated at >£2 billion per annum due to productivity losses combined with the costs of prevention and/or therapy (Williams et al., 1999; Dalloul and Lillehoj, 2006). In-feed chemoprophylaxis remains the main method of control, but concerns about drug residues in the food chain, widespread drug resistance, and legislative restrictions on the prophylactic use of ionophore antibiotics in poultry, all have major impacts (Chapman, 1997; Jenkins et al., 2017b). Oral vaccination with formulations of live wild-type or attenuated parasites is highly effective, but lack of cross-protective immunity means vaccines need to include vaccine lines of all *Eimeria* spp. that pose a risk during the lifetime of the chicken. As each vaccine line requires independent passage through pathogen-free chickens, vaccines are relatively costly to make. The uptake of commercial vaccines within the global broiler sector remains low, and there is an urgent need to develop cheaper, scalable vaccines (Blake and Tomley, 2014).

Previously we have shown that transgenic populations of *Eimeria* parasites can express and deliver vaccine antigens from *Campylobacter jejuni*, infectious bursal disease virus (IBDV) and infectious laryngotracheitis virus (ILTV) to chickens (Clark et al., 2012; Marugan-Hernandez et al., 2016). This technology has the potential to streamline current chicken coccidiosis vaccines, by reducing the complexity of existing formulations (with up to eight different parasites) to a single or small number of parasite lines that express immunoprotective antigens from all the relevant species of *Eimeria*. We are now able to demonstrate the first important step in the development of such a multivalent live vaccine by showing that expression in *Eimeria tenella* of a single antigen (*EmAMA1*)

from *Eimeria maxima* is sufficient to induce statistically significant partial protection against challenge with *E. maxima* oocysts that is broadly equivalent to that obtained using the same antigen in other vaccine platforms.

To date no cross-protective parasite antigens have been described, but several induce between 30 and 70% immune protection against homologous *Eimeria* challenge when tested experimentally with diverse delivery platforms (Blake and Tomley, 2014). The most promising antigens are derived from early endogenous stages of the *Eimeria* lifecycle (sporozoite and first generation schizont), which correlates with the findings observed in naturally infected chickens, where these stages induce the strongest anti-*Eimeria* immunity (McDonald et al., 1986, 1988). Several of these antigens have critical roles in host-parasite interactions including proteins that traffic to the parasite surface and beyond via the secretory microneme (MIC) organelles such as MIC2 (Sathish et al., 2011), MIC3 (Lai et al., 2011), MIC4 (Witcombe et al., 2004), and apical membrane antigen (AMA)1, which achieves around 45% immunoprotective capacity against homologous challenge with *E. maxima* (Blake et al., 2011; Li et al., 2013) or *Eimeria brunetti* (Hoan et al., 2014).

AMAs are a family of proteins expressed by phylogenetically distinct classes of obligate intracellular apicomplexans including species of *Plasmodium* and the zoonotic coccidian *Toxoplasma gondii*, where they are regarded as strong vaccine candidates (Remarque et al., 2008; Zhang et al., 2015). These proteins are critical for the formation and maintenance of the moving junction, a stable focus of adhesion between parasite and host cell membranes through which the parasite glides into its intracellular vacuole (Aikawa et al., 1978; Besteiro et al., 2009; Tyler and Boothroyd, 2011). *Plasmodium falciparum* AMA1 exhibits very high levels of genetic polymorphism which poses a major challenge for vaccine development, hence recent trials have included co-administration of several *PfAMA1* allelic variants (Faber et al, 2016). In contrast, a recent study of genetic variation in *E. tenella* identified little intrinsic polymorphism at the *ama1* locus ETH\_00007745 (ToxoDB, (Blake et al., 2015)) and we now report similar findings for the locus in *E. maxima* (GenBank accession number FN813221).

Proteomic analysis of *E. tenella* initially identified two dominant stage-regulated AMAs specific for either sporozoites (*EtAMA1*, ToxoDB Accession number ETH\_00007745) or merozoites (*EtAMA2*, ToxoDB Accession number ETH\_000048600) (Lal et al., 2009; Oakes et al., 2013). More recently, genomic and phylogenetic analysis defined four classes of AMA paralogues (AMA1-4) that are conserved across haemosporine and coccidian apicomplexans, including *Eimeria* (Parker et al., 2016). Polyclonal antiserum against recombinant *EtAMA1* were shown to have inhibitory effects on sporozoite invasion of cultured cells (Jiang et al., 2012), however the serum reacted on western blots and IFAT with both sporozoite and merozoite antigens, suggesting either that *EtAMA1* protein is expressed by both parasite stages or that the serum recognises more than one AMA paralogue. In this paper we have clarified the stage-specific nature of these two dominant AMAs expressed by *E. tenella* and show that whilst sporozoite-specific *EtAMA1* induces partial immunoprotection against homologous parasite challenge, merozoite-specific *EtAMA2* does not.

The focus of the present work was thus two-fold. First, to characterise and test the potential of the two most abundantly expressed AMA paralogues of *E. tenella* (*EtAMA1* and *EtAMA2*) as recombinant vaccines against homologous challenge (*E. tenella*) in chickens. Subsequently, armed with the information that vaccination with *EtAMA1*, but not *EtAMA2*, significantly reduces the ability of *E. tenella* challenge parasites to replicate, we generated transgenic populations of *E. tenella* that express the orthologous sporozoite-specific AMA1 protein from *E. maxima* and tested these as live vectored vaccines, showing that they confer a similar level of partial protection against heterologous challenge (*E. maxima*). These results confirm the vaccine potential of AMA antigens for coccidiosis and offer new opportunities for the development of multivalent vaccines against all the *Eimeria* spp. with relevance to the poultry industry.

## 2. Materials and methods

### 2.1. Parasites and birds

Oocysts of the Houghton (H) and Wisconsin (Wis) strains of *E. tenella*, and the Weybridge (W) strain of *E. maxima* were propagated by regular in vivo passage through three weeks old Light Sussex or Lohmann Selected Leghorn (LSL) chickens reared under specific pathogen-free conditions (Long et al., 1976). Oocysts were recovered and sporulated, and sporozoites were purified through columns of nylon wool and DE-52, using standard methods (Shirley et al., 1995). Second generation merozoites from *E. tenella* parasites were recovered from the intestines of chickens 112 h p.i. by trypsinisation (Shirley et al., 1995). Apical organelles (micronemes and rhoptries) were isolated from sporozoites and purified by sucrose-density ultracentrifugation as previously described (Kawazoe et al., 1992).

### 2.2. Sequence analysis

*EtAMA1* (ToxoDB Accession number ETH\_00007745) and *EtAMA2* (ToxoDB Accession number ETH\_00004860) protein sequences were aligned using the Multiple Sequence Comparison in the Log-Expectation tool (MUSCLE, [www.ebi.ac.uk](http://www.ebi.ac.uk)) and edited using the BioEdit software v7.1.1. This program was also used to estimate the identity and similarity percentages through the BLOSUM62 matrix.

### 2.3. Isolation of nucleic acids and proteins, and synthesis of complementary DNA from oocysts

Genomic DNA (gDNA) and total RNA were extracted from oocysts using the TRIzol® reagent (Invitrogen, Paisley, UK) as detailed previously (Marugan-Hernandez et al., 2016). Protein extracts from sporozoites and merozoites were obtained following standard procedures (Tomley, 1994). Complementary DNA (cDNA) was also generated using the SuperScript II® reverse transcriptase and random hexamer primers (Invitrogen) as previously described (Marugan-Hernandez et al., 2016).

### 2.4. Recombinant expression of *EtAMA1* and *EtAMA2*

The extracellular domains of *EtAMA1* (ser16-gly446, ETH\_00007745 in ToxoDB) and *EtAMA2* (cys15-gly452, ETH\_00004860) were amplified from sporozoite or merozoite cDNA by PCR using the Platinum Taq DNA Polymerase High Fidelity® (Invitrogen) and the following primers (5'-3'): ATAGGATCCGAGCTGCGCAGGGCCGGCAGCA and GCGAAGCTTTTAACCGCCCCCTTTAGACTCGC for *EtAMA1*, and ATAGGATCCGTGCATCAGTGCCGTGGCGGCA and CGCAAGCTTTTAGCCGAAGCTAACGCCCAGGG for *EtAMA2* (Sigma-Aldrich, Suffolk, UK). Primers incorporated a *Bam*HI site at the 5' end (underlined), and a TAA stop codon and *Hind*III site at the 3' end. The digested, gel purified (Qiagen, West Sussex, UK) PCR products were ligated to a pET32b (+) vector that had been previously linearised by double digestion with *Bam*HI and *Hind*III (New England BioLabs, Hertfordshire, UK). *EtAMA1* and *EtAMA2* sequences were ligated to pET32b (+) by T4 ligase (Promega, Hampshire, UK), propagated in *Escherichia coli* XL1-Blue competent cells (Stratagene, California, USA), purified using the QIAprep Spin Miniprep kit (Qiagen), and checked by restriction analyses using *Bam*HI and *Hind*III. Recombinant *EtAMA1* and *EtAMA2* were produced in transformed BL21(DE3)pLysS cells (Novagen, Hertfordshire, UK) and purified using HisTrap FF purification columns (GE Healthcare, Buckinghamshire, UK) following standard procedures (Blake et al., 2011). Proteins were dialysed extensively against PBS before use.

## 2.5. Generation of polyclonal antibodies against *EtAMA1* and *EtAMA2*

Hyperimmune antisera were raised in mice (*EtAMA1* and *EtAMA2*), chickens (*EtAMA1* only), or rabbits (*EmAMA1*) against HisTrap FF purified recombinant proteins (see Section 2.4 and (Blake et al., 2011)). Groups of three or four animals were immunised three times at fortnightly intervals with between 10 and 50 µg of soluble antigen suspended in 100 µl of PBS and mixed with an equal volume of Titremax gold (first immunisation) or Freund's incomplete adjuvant (second and third immunisations) (Kawazoe et al., 1992). Animals were bled 7 days after the second and third immunisations and all sera screened by western blotting against solubilised whole sporozoite and merozoite proteins.



## 2.6. Indirect immunofluorescence (IFAT)

Purified *E. tenella* sporozoites were fixed in 3% paraformaldehyde in PBS for 10 min then 20  $\mu$ L spots of the suspension were dried onto glass coverslips. Dried parasites were permeabilised by immersion in ice-cold methanol for 5 min, followed by rehydration in PBS for 20 min. IFAT was carried out as described (Bumstead and Tomley, 2000) using antiserum to *EtAMA1* or *EtAMA2*. The DNA stain DAPI (4', 6'-diamidino-2 phenylindole) was included in the second antibody incubation step. Coverslips were air dried, mounted on Vectashield (Vector Laboratories, Peterborough, UK) and photographed at  $\times 1,000$  magnification under UV light using a Leitz fluorescence microscope. Alternatively, tissue sections of infected caeca containing second generation schizonts as used for merozoite harvest were de-waxed and pressure cooked. All samples were treated with PBS-1% BSA to block non-specific staining, exposed to primary antibody for 1 h and then, after washing, were incubated with FITC-conjugated anti-chicken or anti-mouse-IgG (Sigma-Aldrich). Some samples were counterstained with DAPI prior to examination. Coverslips and sections were examined with a Zeiss Axioskop microscope, and pictures were taken with a cooled CCD camera using Improvision Openlab software.

## 2.7. In vitro secretion assays

Freshly excysted *E. tenella* H sporozoites were purified, resuspended at  $10^8$ /ml in HAMs F9 media (Sigma-Aldrich) with or without 1% FCS supplementation (Sigma-Aldrich), and incubated at 41  $^{\circ}$ C. Supernatants were removed at 0, 10 and 30 min post-incubation, clarified by centrifugation at 10,000  $g$  for 10 min at 4  $^{\circ}$ C, and then stored at -20  $^{\circ}$ C until examined by SDS-PAGE and western blotting (as described in Section 2.10). Rabbit antiserum against the *E. tenella* microneme protein 3 (*EtMIC3*) was used as a positive control for secretion (Lai et al., 2011) and against the *E. tenella* heat shock protein 70 (*EtHSP70*) protein as a control for inadvertent sporozoite lysis (Dunn et al., 1995; Bumstead and Tomley, 2000).

## 2.8. *In vitro* sporozoite inhibition assays

Wells of 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) were seeded with 100 µl of a suspension of Madin Darby Bovine Kidney (MDBK) cells ( $6 \times 10^5$ /ml) in HAMs F-12 medium (Sigma-Aldrich) and incubated at 41 °C until the cells were settled and monolayers reached ~85% confluency. Freshly hatched *E. tenella* sporozoites were suspended at  $10^6$ /ml in HAMs F-12 supplemented with dilutions of anti-EtAMA1 or anti-EtAMA2 mouse sera (1/50 to 1/800). Alternatively, freshly plated MDBK cell monolayers were exposed to medium supplemented with dilutions of EtAMA1 or EtAMA2 recombinant protein (1 to 0.625 µg/ml). For all treatments, parasites or cells were incubated in triplicate for 15 min at room temperature. A mouse antibody against parasite enolyl reductase (ENR) and the recombinant thioredoxin protein were included as control treatments for each respective protocol. The incubation medium was removed from each well, replaced with 200 µl of the appropriate pre-incubated sporozoite suspension and the infected monolayers returned to 41 °C. Infected cultures were incubated for 48 h and the overall inhibition of parasite growth measured by comparison of the incorporation of labelled uracil into untreated cultures, using well established procedures (Schmatz et al., 1986). In this case infected monolayers were spiked with 1 µCi per well of [3H] uracil at 24 h p.i., then returned to 41 °C for a further 24 h at which time cells were lysed and tritium incorporation was counted. Data were analysed using the Tukey's multiple comparisons test from the GraphPad Prism software (version 7.02).

## 2.9. *In vivo* EtAMA1 and EtAMA2 immunisation trials

Coccidia-free Light Sussex chickens were housed within specific pathogen-free (SPF), coccidia-free conditions in groups of four (experiment one) or five (experiment two). Chickens were immunised at one, three and 5 weeks of age by s.c. injection with 100 µg of the corresponding vaccine antigen (Table 1). The first two preparations were made up to a volume of 0.5 ml and emulsified with 0.5 ml of TiterMax adjuvant (Sigma-Aldrich) except for Group 4, where 0.25 ml of each antigen was emulsified separately with 0.25 ml of TiterMax adjuvant and administered together. The last

preparations were emulsified with Freund's incomplete adjuvant (Sigma-Aldrich). At 7 weeks of age, birds were moved into single cages and each chicken was given an oral dose of 250 sporulated *E. tenella* H strain oocysts to quantify the effect of the vaccine on parasite replication. Daily faecal samples were collected from each bird from 5 to 12 days after challenge and the number of oocysts excreted in the faeces calculated by counting of samples following a standard protocol (Long et al., 1976). Data were analysed by one-way ANOVA with a Tukey's post-hoc test using the GraphPad Prism software (version 7.02).

#### 2.10. SDS- PAGE and western blot

Proteins were electrophoresed through NuPAGE 4–12 % Bis-Tris gels (Invitrogen) in Laemmli loading buffer (Sigma) using the XCell SureLock Mini-Cell Electrophoresis System (Invitrogen), and then either stained using Coomassie brilliant blue R-250 (Bio-Rad, Hertfordshire, UK) or transferred to nitrocellulose membranes (GE Healthcare) in a semi-wet system using the XCell II Blot Module (Invitrogen). Non-specific binding sites were blocked by overnight incubation in 3% BSA (Sigma-Aldrich) in Tris-buffered saline (TBS)-Tween 0.1%, and then filters were probed with either mouse or chicken antibodies, diluted in blocking solution, followed by horseradish peroxidase (HRP) conjugated anti-mouse/chicken second antibodies (Merck Millipore, Hertfordshire, UK). Bound antibody was visualised by enhanced chemiluminescence (ECL) using Luminata Forte Western HRP substrate (Merck Millipore) and a G:BOX coupled with GeneSnap 7.12 software (Syngene, Cambridge, UK).

#### 2.11. *Eimeria maxima* AMA1 amplification and cloning

The coding sequence of the *E. maxima* AMA1 ectodomain (*EmAMA1*, his32-phe446, GenBank Accession number FN813221.1) was amplified from the pET32b-*EmAMA1* plasmid (Blake et al., 2011) and flanked with *Xba*I restriction sites (underlined) by PCR using the primers (5'-3'): GCTCTAGACACCAGGGTCACACAGAA and GCTCTAGAAAGCCGCTTCACACCG (Sigma-Aldrich). A 1,269 bp fragment was amplified using the Platinum Taq DNA Polymerase High Fidelity® (Invitrogen). The

PCR product was resolved by agarose gel electrophoresis, manually excised under UV light and purified using the MinElute Gel Extraction kit (Qiagen). Then, it was cloned using the pGEM®-T Easy vector system (Promega), propagated in *E. coli* XL1-Blue competent cells (Stratagene), purified using the QIAprep Spin Miniprep kit (Qiagen), and sequenced (GATC Biotech, London, UK) as described by the manufacturers. Sequence analysis was done using CLC Main Workbench (Qiagen).

## 2.12. Plasmid constructs for transgene expression of *EmAMA1* in *E. tenella*

Three different core plasmids were used for *E. tenella* transfection as previously described (Marugan-Hernandez et al., 2017). In brief, all the plasmids carried the mCitrine reporter downstream of the *E. tenella* microneme protein (*EtMIC*) 1 5' promoter region, and the mCherry reporter downstream of the 5' *EtMIC2* promoter region, preceded by the *XbaI* restriction site, and flanked with varying combinations of two delivery signals: the signal peptide of the *EtMIC2* protein (SP2), and the glycosylphosphatidylinositol anchor of the *EtSAG1* protein (GPI) (Fig. 1A-C).

The coding sequence of the *EmAMA1* ectodomain was cloned into the *XbaI* site of the three core constructs and therefore fused to the mCherry reporter (Fig. 1). For this purpose, the pGEM-T-*EmAMA1* plasmid was digested with *XbaI* (New England BioLabs) following the manufacturer's protocol, and the *XbaI-EmAMA1-XbaI* insert was purified from agarose gels as described above. The three core constructs were also digested with *XbaI* (single restriction site), treated with calf intestinal alkaline phosphatase (New England BioLabs) according to the manufacturer's instructions, and purified from agarose gels. The ligation of the *EmAMA1* sequence into the core plasmids was carried out using the T4 ligase (Promega) as detailed by the manufacturer. The three new constructs were also propagated in XL1-Blue competent cells, mini-prepped, characterized by restriction analyses to determine the insertion directionality using the *NdeI* enzyme (New England BioLabs), and sequenced to check that the insert reading frame had not been altered by cloning. Final plasmids were prepared for transfection using a Midi Prep Kit (Qiagen), digested for linearization with *ScaI* (New England

BioLabs), precipitated in ethanol-sodium acetate (Marugan-Hernandez et al., 2017) and quantified by NanoDrop (Thermo Scientific).

### 2.13. Drug selection of mutant parasites

A plasmid carrying the mutant *Toxoplasma gondii* dihydrofolate reductase–thymidylate synthase (DHFR-TSm2m3) gene that confers resistance to pyrimethamine (Fig. 1D) (Clark et al., 2008) was also prepared for co-transfection using a Midi Prep Kit, digested for linearization with *PsiI* (New England BioLabs), precipitated in ethanol-sodium acetate and quantified by NanoDrop.

### 2.14. Preparation of *E. tenella* Wis sporozoites expressing EmAMA1 protein

Transfection of freshly hatched *E. tenella* Wis strain sporozoites was carried out by restriction enzyme-mediated integration (REMI) using 16-well strips and the programme EO114 of the Nucleofector 4D (Lonza, Basel, Switzerland) as described previously (Marugan-Hernandez et al., 2016). Briefly,  $1 \times 10^6$  sporozoites were electroporated in duplicate with 10  $\mu$ g of *ScaI* and *PsiI*-digested plasmids (carrying the EmAMA1 sequence with different delivery signals, and the DHFR-TSm2m3 resistance gene, respectively) together with 6 U of the *ScaI* and *PsiI* restriction enzymes in Lonza buffer P3. Shocked parasites were left for 20 min at room temperature in RPMI medium (Sigma-Aldrich), and then sporozoite survival was estimated by microscopy using 0.02% Trypan blue dye (Invitrogen) (Marugan-Hernandez et al., 2017). In order to obtain stable transgenic populations, duplicated wells were pooled together post-transfection and used to infect two birds by the cloaca ( $0.75 \times 10^6$  sporozoites/bird). After 24 h, birds were in-feed supplemented with pyrimethamine for 6 days (150 ppm, Sigma-Aldrich) (Clark et al., 2008). Oocysts were harvested 7 days after infection, sporulated and used for a subsequent in vivo passage after population enrichment for fluorescent parasites by fluorescence-activated cell sorting (FACS) (FACS Aria III, BD, Berkshire, UK) (Marugan-Hernandez et al., 2016).

Transcription of the *EmAMA1* W strain coding sequence was determined by reverse transcription PCR (RT-PCR) using cDNA isolated from the three stable transgenic populations as described above and employing the primers used for *EmAMA1* cloning. To confirm the absence of gDNA in the cDNA samples, primers for the *EtActin* gene were designed across an intron such that gDNA contamination of cDNA would result in amplification of two fragments (358 and 99 bp; 5' to 3': TTGTTGTGGTCTTCCGTCA and GAATCCGGGGAACATAGTAG, Sigma-Aldrich; from (Marugan-Hernandez et al., 2016)). The *EmAMA1* sequence was amplified by PCR using the Platinum Taq DNA Polymerase (Invitrogen), and the corresponding cDNA at 1/10 dilution. The PCR product was resolved by agarose gel electrophoresis.

Expression of the *EmAMA1* protein fused with mCherry was confirmed by microscopy for the fluorescent reporter using the fluorescent microscope DMI3000B – DCF365FX (Leica Microsystems, Milton Keynes, UK). Micrographs of sporulated transgenic oocysts were collected using the SP5 confocal microscope and the Leica Application Suite Advanced Fluorescence software (Leica Microsystems). Image processing was performed using ImageJ software (NCBI, <http://rsb.info.nih.gov/ij/>).

#### 2.15. *In vivo* immunisation trial of *E. tenella* Wis parasites expressing *EmAMA1*

A total of 42 White Leghorn line 151 chicks (highly susceptible to *E. maxima* infection, (Bumstead and Millard, 1992; Smith et al., 2002)); purchased from the National Avian Research Facility, Edinburgh, UK) were split into six groups of seven in independent wire-floored cages (Table 2). One (Group 1, *Et[EmAMA1-Ch]*) and two (Group 3, *Et[SP2-EmAMA1-Ch-GPI]*) chicks were removed prior to the start of the vaccination trial for husbandry reasons. Birds from groups 1 to 4 were immunised by oral gavage at days 2, 8, 14 and 21 of age with 100, 500, 3,000, and 5,000 FACS enriched transgenic *EmAMA1 E. tenella* sporulated oocysts, respectively, to mimic parasite recycling, whereas birds from groups 5 to 6 were inoculated with sterile PBS following the same scheme. At day 30, birds from groups 1 to 5 were challenged with 300 oocysts of the *E. maxima* W strain to quantify the effect

of the vaccine on parasite replication. Body weights and serum samples were collected at days 29 (8 days after the final vaccination) and 36 (6 days post-challenge) of age to determine the safety, immunogenicity, and efficacy of the recombinant vectored vaccines. Also, individual faecal samples were collected from days 35 to 39 (5 - 8 days post-challenge) to determine the total oocyst output after *E. maxima* challenge (Shirley et al., 2005). Data were analysed by one way ANOVA with a Tukey's post-hoc test using the GraphPad Prism software (version 7.02).

#### *2.16. Analysis of immune responses elicited after vaccination with E. tenella* Wis parasites expressing *EmAMA1* and after challenge with *E. maxima* W

Serum samples collected on day 29 were used to detect IgY responses against *EmAMA1* protein by western blot. All sera (1/50 to 1/400 dilutions) were directed against nitrocellulose membranes transferred with recombinant *EmAMA1* or with *E. maxima* protein extracts as detailed in Section 2.10. For *E. maxima*-based western-blots, rabbit anti-*EmAMA1* antiserum was used as the positive control to detect native *EmAMA1* protein in oocyst extracts and compare its recognition pattern with those obtained using the test serum samples.

In addition, serum samples collected on day 29 and 36 were used to quantify serum IL-10 and IFN- $\gamma$ . IL-10 ELISA was performed as described previously (Wu et al., 2016). Briefly, assay plates (Nunc Immuno MaxiSorp) were coated overnight at 4 °C with 3  $\mu$ g/ml of capture antibody, incubated with 50  $\mu$ l of two-fold serially diluted standards or serum samples, and incubated with 1  $\mu$ g/ml of biotinylated detection antibody. The plates were incubated with streptavidin-HRP and developed with 1-Step™ Turbo TMB substrate solution (Thermo Scientific). The reaction was stopped by adding 50  $\mu$ l of 2N sulphuric acid after 10 min, and assays were read at 450 nm. IFN- $\gamma$  levels were measured using the IFN- $\gamma$  chicken matched antibody pair (Thermo Scientific) following the manufacturer's instruction. Data were analysed by one-way ANOVA with a Tukey's post-hoc test using GraphPad Prism software (version 7.02). Associations between oocyst shedding and cytokine levels were estimated using the Pearson correlation coefficient with the same software.

## 2.17. Genetic characterisation of *E. maxima* and *E. tenella* apical membrane antigens

Gene models for the *E. tenella* apical membrane antigens 2-4 were downloaded from ToxoDB (Gajria et al., 2008; Parker et al., 2016) and used to identify candidate *E. maxima* orthologues using tBLASTx with default parameters in ToxoDB. Coding sequences for *EmAMA1* and *EtAMA1* were accessed from GenBank and ToxoDB (FN813221 and ETH\_00007745). Transcription profiles were accessed for all *E. tenella* genes using published RNAseq data (Reid et al., 2014; Walker et al., 2015). Signatures of selection were assessed using mean Ka/Ks scores (*Eimeria acervulina*, *E. brunetti*, *E. maxima*, *Eimeria mitis*, *Eimeria necatrix* and *Eimeria praecox* compared with *E. tenella*, (Reid et al., 2014)). For *E. maxima*, additional measures of genetic diversity were calculated using 18 coding sequences derived from India ( $n=3$ ), Nigeria ( $n=2$ ), Spain ( $n=1$ ) and the UK ( $n=12$ ) available under the GenBank accession numbers **FN813221-2**, **LN626985-91** and **LT900485-LT900492**. Analyses undertaken using DnaSP v5.10 (Librado and Rozas, 2009) included identification of the number of variant sites (S) and numbers of non-synonymous (dN) or synonymous (dS) substitutions. Diversity was defined by calculating the average nucleotide difference (k) and nucleotide diversity ( $\pi$  with the Jukes Cantor correction). Allelic diversity was defined by calculating the number of haplotypes (H) and the associated haplotype diversity (Hd). Neutrality was assessed using Tajima's D and Fu and Li's D\* and F\* tests with the total number of mutations and significance set at  $P < 0.05$ . All analyses were performed as described previously and compared with data published for EtAMA1 (Blake et al., 2015).

## 2.18. Ethical statement

This study was carried out in strict accordance with the Animals (Scientific Procedures) Act 1986, an Act of Parliament of the United Kingdom. All animal studies and protocols were approved by the Royal Veterinary College Animal Welfare & Ethical Review Body (London, UK) and the United Kingdom Government Home Office under specific project licence. The laboratory work involving genetic modified organisms (GMO) was conducted under authorization GM9708.1, administered by the UK Health and Safety Executive.



### 3. Results

#### 3.1. Expression of *EtAMA* genes is stage-regulated

The protein sequences of *EtAMA1* and *EtAMA2* were compared using the BLOSUM62 matrix, and displayed low identity and similarity scores (0.338 and 0.474, respectively) (Supplementary Fig. S1). Samples of unsporulated oocysts (UO,  $1.25 \times 10^5$ /well), sporulated oocysts (SO,  $1.25 \times 10^5$ /well), sporozoites (Sz,  $1 \times 10^6$ /well) and second generation merozoites (Mz2,  $1 \times 10^6$ /well) were probed with hyperimmune mouse serum against *rEtAMA1* or *rEtAMA2*. *EtAMA1* was detected in the sporulated oocyst and sporozoite lanes and was absent from the merozoites. Conversely, *EtAMA2* was detected only in merozoites (Fig. 2A). Subsequent comparison of RNAseq datasets representing UO, SO, Sz, Mz2 and gametocyte (Gam) lifecycle stages confirmed these findings, with additional evidence of stage-specific transcription for *EtAMAs* 1-4 (Table 3). Mean Ka/Ks ratios of less than 1.0 were reported for *AMA1* and *AMA4*, indicating purifying or stabilising selection.

Samples of sporozoites and gradient-enriched preparations of sporozoite micronemes and rhoptries were probed with hyperimmune mouse and chicken sera raised against the same preparation of *rEtAMA1*. The mouse antiserum recognised a single microneme protein of an apparent molecular mass of ~63 kDa, whereas the chicken antiserum additionally reacted with a protein in the rhoptry fraction of ~60 kDa (Fig. 2B). *EtMIC3* antiserum demonstrated that the rhoptry fraction was not significantly contaminated with micronemes, and *EtHSP70* antiserum (a common contaminant of rhoptry fractions, (Dunn et al., 1995)) demonstrated that the microneme fraction was not contaminated with rhoptries.

Immunofluorescent antibody staining of sporozoites with mouse anti-*rEtAMA1* showed an apical localisation within the zoite (Fig. 2C), whereas mouse anti-*rEtAMA2* showed clear apical staining of mature merozoites in caecal sections harvested 114 h p.i. with *E. tenella* H strain (when second generation schizogony is underway) (Fig. 2C). Some sections were counterstained with antibodies against the merozoite surface antigen *EtSAG4*, to delineate the outline of merozoites within schizonts. Others were counterstained with antibodies against *EtMIC2* to delineate the micronemes.

Interestingly, *EtAMA2* only partially co-localised with *EtMIC2*, indicating that it may not all be localised within the microneme organelles or alternatively that the two proteins may be differentially distributed within the microneme population (Fig. 2C). A similar phenomenon was previously noted when co-staining for *EtAMA1* and *EtMIC3* (Lai et al, 2011).

### 3.2. *EtAMA1* is secreted by sporozoites and involved in host-cell sporozoite invasion

Freshly excysted and purified sporozoites were incubated with or without FCS for up to 30 min. In the absence of FCS, *EtAMA1* was secreted at a low level by sporozoites whereas FCS supplementation induced rapid secretion into the culture supernatant in a manner typical of *E. tenella* microneme proteins (Fig. 2D) (Lai et al., 2011). Pre-treatment of MDBK cells or *E. tenella* sporozoites with, respectively, recombinant *EtAMA1* (0.5 to 1 µg) or mouse anti *rEtAMA1* (1/800 to 1/50 dilution), resulted in significant reductions in parasite-specific uracil uptake ( $P < 0.05$ , Tukey's post hoc test), indicative of reduced invasion and/or parasite replication. In contrast, treatment with recombinant *EtAMA2*, recombinant thioredoxin, anti *rEtAMA2* serum, or anti-ENR serum did not affect uracil uptake (Fig. 2E).

### 3.3. Recombinant *EtAMA1* induces an immune response protective against *E. tenella* challenge

In experiment 1, vaccination with *rEtAMA1* induced a significant reduction in oocyst output after challenge with *E. tenella* ( $P < 0.05$ , one way ANOVA test), reducing oocyst shedding by 77.4% compared with the non-vaccinated control, whereas no significant differences were found between chickens vaccinated with *rEtAMA2* and non-immunised animals ( $P > 0.05$ , one way ANOVA test) (Fig. 3). Similarly, in experiment 2, vaccination with *rEtAMA1* or with combined *rEtAMA1+rEtAMA2* induced a significant reduction in oocyst output after challenge with *E. tenella* ( $P < 0.05$ , one way ANOVA test), reducing oocyst shedding by 76 and 66% compared with the non-vaccinated control, respectively. Again, no significant differences were found between chickens vaccinated with *rEtAMA2* and non-immunised animals ( $P > 0.05$ , one way ANOVA test) (Fig. 3).

3.4. *Eimeria tenella* can express the foreign antigen *EmAMA1* and modify its trafficking when complemented with appropriate delivery signals

Three populations of transgenic *E. tenella* parasites expressing *EmAMA1* were generated by transfection and stabilised by sequential passage through chickens medicated with pyrimethamine (Fig. 4A). The first round of infection resulted in shedding of 20 to 60×10<sup>6</sup> oocysts/bird 1 week p.i., of which 1 to 2% expressed the mCitrine reporter. FACS enrichment of mCitrine expressing parasites was ~95% efficient and successive in vivo passage of FACS isolated mCitrine-positive parasites under pyrimethamine selection improved the transgenic proportion of each population from 1 to 2% at passage 1, to 16 to 42% at passage 3 (Fig. 4A).

Transcription of *EmAMA1* mRNA was confirmed by RT-PCR in all three transgenic populations in the absence of gDNA contamination (Fig. 4C). In addition, expression of the *EmAMA1* protein was indicated by detection of the fused mCherry reporter by fluorescence microscopy (Fig. 4B). In the three populations, FACS enriched parasites expressed mCitrine as a cytosolic protein, whereas the expression pattern of mCherry differed among them. Parasites not complemented with delivery signals expressed the mCherry as a cytosolic protein which co-localised with mCitrine (*EmAMA1-Ch* in Fig. 4B). In contrast, populations complemented with the SP2 sequence secreted the mCherry reporter into the sporocyst cavity (*SP2-EmAMA1-Ch* and *SP2-EmAMA1-Ch-GPI* in Fig. 4B). Furthermore, complementation with the GPI-anchor additionally induced the anchorage of mCherry onto the sporozoite surface (*SP2-EmAMA1-Ch-GPI* in Fig. 4B). In all populations both mCitrine and mCherry were present within cytosolic aggregates, which in some cases were co-localised (Fig. 4B).

3.5. Vaccination with transgenic *E. tenella* expressing *EmAMA1* protects against challenge by the antigen donor *E. maxima* without eliciting specific humoral responses, but reducing serum IL-10 levels

Vaccination with live *E. tenella* parasites did not result in significant differences in weights between groups prior to challenge ( $P > 0.05$ , one way ANOVA test) indicating no detrimental effect of the vector. Similarly, no differences in body weight gain were found between groups after challenge

with *E. maxima* W strain, as the dose used was deliberately low in order to accurately determine the vaccination effect on parasite replication ((Blake et al., 2006);  $P > 0.05$ , one way ANOVA test) (Supplementary Fig. S2A and B). Vaccination with all three transgenic *E. tenella* populations expressing *EmAMA1* resulted in significant reductions in oocyst output after challenge with *E. maxima* ( $P < 0.05$ , one way ANOVA test), ranging from 45 to 55% compared with the non-vaccinated and the challenged groups (Fig. 5A). Pairwise comparisons did not show statistical differences between the three vaccinated groups ( $P > 0.05$ , one way ANOVA test).

Serum samples collected 8 days after final vaccination (day 29) showed variable antibody reactivity against *rEmAMA1* protein within experimental groups, and no reactivity against native *EmAMA1* in *E. maxima* solubilised protein extracts, suggesting that the observed reduction in parasite replication after challenge was not mediated by specific humoral responses against *EmAMA1* (Fig. 5B). Similarly, we did not detect enhanced levels of IFN- $\gamma$  in serum 8 days after final vaccination (day 29) or 6 days after challenge (day 36), with equally low concentrations across all groups (under 60 pg/ml,  $P > 0.05$ , one way ANOVA test) (Fig. 5C). At day 29, all groups displayed low levels of serum IL-10 ( $P > 0.05$ , one way ANOVA test) (Fig. 5C) and at 6 days post-challenge (day 36) the average IL-10 serum levels were lower in all groups immunised with transgenic parasites compared with non-vaccinated and challenged birds (Fig. 5C). However this reduction was only significant in groups vaccinated with *Et*[SP2-*EmAMA1*-Ch] and *Et*[SP2-Ch-GPI] parasites ( $P < 0.05$ , one way ANOVA test), suggesting that the effect is not mediated by expression of *EmAMA1*. A positive correlation was detected between serum IL-10 levels and oocyst output (64%,  $P < 0.001$ , Pearson correlation test, data not shown).

### 3.6. Sequence diversity for the anticoccidial vaccine candidate *EmAMA1* is limited and comparable to the *E. tenella* orthologue

Comparison of 56 *E. tenella* AMA1 coding sequences previously revealed limited antigenic diversity and a largely neutral signature of selection (Blake et al., 2015). Here, comparison of 18 *EmAMA1* coding sequences from parasites sampled in four countries, across three continents,

revealed a comparably low level of diversity (Table 4). Specifically, nine *EmAMA1* cDNA haplotypes were identified but total nucleotide diversity was low and no significant signatures of selection were detected. Analysis of the 1,617 bp coding sequence alignment revealed 10 nucleotide substitutions, of which three were non-synonymous. Substitutions of histidine to leucine (amino acid position 81), isoleucine to methionine (position 133) and glutamic acid to aspartic acid (position 393) were detected, of which the latter was located close to the glutamic acid to valine (position 386) substitution described previously in the *EtAMA1* amino acid sequence (Blake et al., 2015).

#### 4. Discussion

In the present work we have characterised the two most highly expressed AMAs of *E. tenella*, *EtAMA1* (ToxoDB Accession number ETH00007745) and *EtAMA2* (ToxoDB Accession number ETH00004860) and demonstrate, to our knowledge for the first time, that vaccination with sporozoite-specific *EtAMA1*, but not merozoite-specific *EtAMA2*, elicits highly significant protection against homologous challenge with *E. tenella*. With this knowledge we went on to establish a proof of concept for the use of *Eimeria* parasites as vaccine delivery vectors for heterologous coccidial antigens, showing that *E. tenella* parasites expressing *EmAMA1* antigen from *E. maxima* are able to induce partial protection in all the vaccinated birds against heterologous challenge infection with *E. maxima*.

A polyclonal mouse serum raised against *rEtAMA1* detected native *EtAMA1* in sporulated oocysts and sporozoites, but not in merozoites, and conversely mouse serum against *rEtAMA2*, detected native *EtAMA2* solely in merozoites. These results agree with previous proteomic and transcriptomic data and confirm that these two *EtAMA* paralogues are tightly stage-regulated (Lal et al., 2009; Reid et al., 2014). Interestingly a second antibody raised against *rEtAMA1* in chickens detected an additional sporozoite protein (Fig. 2B) indicating the potential for cross-reactivity of anti-AMA1 antibodies. This additional protein was not identified, but it could potentially be *EtAMA3* or *EtAMA4*, both of which are expressed in the sporozoite stage (Table 3). Importantly, we showed that the mono-specific mouse anti-*rEtAMA1* serum as well as *rEtAMA1* protein were both efficient at

reducing sporozoite invasion of cultured cells, in agreement with previous work (Jiang et al., 2012), whereas anti-*rEtAMA2* and *rEtAMA2* protein had no effect on parasite invasion, again indicating the highly stage-specific nature of the two paralogues. The possession of four AMA paralogs in *T. gondii* is proposed to confer exceptional molecular plasticity at the parasite–host-cell interface (Poukchanski et al., 2013; Lamarque et al., 2014; Parker et al., 2016). In this work, we identified the *EtAMA2* gene in merozoite-derived expressed sequence tags (ESTs) (data not shown) supporting previous observations based on transcriptomic data (Reid et al., 2014), and confirmed that *EtAMA2* protein is found in merozoites by western blotting and IFAT experiments (Lal et al., 2009). Intriguingly, RNAseq evidence published by others also indicates *EtAMA2* gene transcription in gametocytes, although the precise function of an AMA paralogue in this stage of the parasite is unclear (Walker et al., 2015). All these findings indicate that *E. tenella* parasites harbour stage-specific AMA proteins that could be relevant during specific phases of the parasite cycle. Interestingly, Jiang and colleagues apparently detected *EtAMA1* protein in merozoites and gametes despite transcriptomic evidence discarding this possibility (Jiang et al., 2012), which could potentially be artefactual and due to their anti-*EtAMA1* serum cross-reacting with alternative *EtAMA* paralogs.

The results obtained in in vitro sporozoite inhibition assays were in line with what was subsequently observed when *rEtAMA1* and *rEtAMA2* were used to immunise chickens. Birds vaccinated with *rEtAMA1*, but not *rEtAMA2*, produced significantly fewer oocysts after challenge with *E. tenella*, and vaccination with combined *rEtAMA1* and *rEtAMA2* did not induce any synergistic effect. Similar promising results have been seen in previous studies where chickens were vaccinated with AMA1 proteins from *E. maxima* (DNA, recombinant protein and Bacille Calmette-Guérin -vectored immunisations) or *E. brunetti* (DNA immunisation) and vaccine efficacy was measured in a variety of ways including improved body weight gains and reduced gut lesions after high dose challenge, or reduced oocyst shedding after low dose challenge (Blake et al., 2011; Li et al., 2013; Hoan et al., 2014).

Given their proven ability to induce robust immunoprotective responses, and the availability of established methods for vaccine administration, the use of live recombinant *Eimeria* vectors as

streamlined vaccines against coccidia in poultry is highly attractive. In earlier studies we demonstrated that vaccination with *E. tenella* Wis strain parasites expressing the CjaA protein from *Campylobacter jejuni* induced significant protection (86-91%) against homologous challenge infection, regardless of the number of immunisations (Clark et al., 2012). Subsequently, we showed that *E. tenella* Wis parasites expressing the vvVP2 protein from infectious bursal disease virus (IBDV), or glycoprotein I from infectious laryngotracheitis virus (ILTV), elicited limited but specific humoral responses when used as vaccines (Marugan-Hernandez et al., 2016). More recently, we carried out extensive work to improve the *Eimeria* vector system by inducing higher expression of the transfected gene and increasing exposure of the transprotein to the host immune system through the use of specific promoters and delivery signals, respectively (Marugan-Hernandez et al., 2017). The promising results obtained with *EtAMA1* and *EmAMA1* subunit vaccines prompted us to test the fitness of this new delivery system (*E. tenella* Wis-based) to express the *EmAMA1* protein from *E. maxima* at high levels and modify the protein trafficking by inducing its secretion and tethering to the sporozoite surface. As expected, all three transgenic populations transcribed the *EmAMA1*-mCherry sequence, expressed the protein at high levels, and displayed differential secretion patterns (Fig. 4). In previous work we showed that *E. tenella* parasites expressing the yellow fluorescent protein (YFPmYFP) can be stabilized after three generations under double selection (Clark et al., 2008). In the present study, the same number of in vivo passages was insufficient to obtain fully fluorescent populations, although all populations displayed distinct increases in the percentage of stable transgenic parasites after three generations (Fig. 4A). We have observed this to be the case for several other co-transfections (data not published), and hypothesise that it could be due to the disruption of the integrated plasmids during sporulation, when meiosis takes place under no drug selection.

Previous studies have shown that the immunological activity of AMA1 in *Plasmodium* parasites relies on its correct folding (Hodder et al., 2001). While we did not check whether *EmAMA1* was properly folded in transgenic populations, we assumed that the use of an *E. tenella*-based vector would support appropriate folding of foreign proteins from other *Eimeria* spp., and according to our

data this seems to have been the case. We observed similar results in a previous study carried out with birds that received single or trickle vaccinations with *E. tenella* Wis parasites expressing EmAMA1 under the control of the *EtAMA1* promoter and displayed a reduction in the oocyst output ranging from 38.4 to 46.9% after challenge with *E. maxima* W (Supplementary Fig. S3). Furthermore, similar cross-strain heterologous protection results were observed when *E. maxima* H strain sporozoites transiently transfected with a bacterial artificial chromosome (BAC) construct carrying the EmAMA1 sequence from the *E. maxima* W strain were used as a vaccine (Blake et al., 2011). All these data offer promising prospects for the future development of anti-coccidial vaccines based on *Eimeria* as a vector.

Interestingly, all three transgenic populations induced similar levels of heterologous protection after challenge with *E. maxima*, suggesting that the *EmAMA1* protein is similarly processed by the host immune system regardless of the delivery signal employed. However, sera collected after vaccination were consistently not reactive against the native *EmAMA1* protein within *E. maxima* protein extracts (Fig. 5B). These findings correlate with our previous study, in which vaccination with transgenic *Eimeria* parasites induced low antibody titres against the transgene (Marugan-Hernandez et al., 2016), and indicate that this vector system may not be suitable when specific humoral responses are needed. The role of the humoral response in conferring resistance to coccidiosis is still debated, but it is clear that antibody titres do not correlate with levels of protection (Lillehoj and Ruff, 1987; Wallach et al., 1992; Dalloul et al., 2003; Lee et al., 2009a, b). Our study supports this view, since all vaccinated groups were able to reduce *E. maxima* replication after challenge despite the absence of reliably detectable levels of antibodies specific for *EmAMA1*.

It is well established that *Eimeria* parasites induce significant increases in IFN- $\gamma$  mRNA levels in the infected tissues (Rothwell et al., 1995; Min et al., 2003), as well as protein levels in serum (Yun et al., 2000) and peripheral blood leucocytes (Breed et al., 1997). However, we detected little or no enhanced serum IFN- $\gamma$  levels compared to the controls at either day 29 (after four oral vaccinations with transgenic *E. tenella*) or day 36 (6 days after challenge with *E. maxima*). We did not expect to see



enhanced levels at day 29, as this was 4 weeks after the primary vaccination, but did expect to see induction of serum IFN- $\gamma$  at day 36, as this is reported to increase in serum at 8 days post challenge with *E. maxima* (Yun et al., 2000). However, an earlier report did not detect serum IFN- $\gamma$  until 20 days following primary and serial exposure to *E. maxima* (Yun et al., 2000), thus the timing may vary according to host strain and parasite dose. Similarly, the trickle vaccination did not affect IL-10 serum levels at day 29 compared to controls (Fig. 5C), and again this is most likely due to the timing of sampling. In contrast, all challenged birds showed increased serum IL-10 levels at day 36, including birds immunised with transgenic parasites. Expressed serum IL-10 was lower in all immunised birds compared to non-immunised birds, irrespective of exposure to *EmAMA1*. Increased serum IL-10 at day 36 in all challenged birds may be explained by exposure to different *Eimeria* spp. at immunisation and challenge (transgenic *E. tenella* and *E. maxima*), effectively indicating different primary infections. The lower serum IL-10 levels in birds immunised using *Et*[SP2-Ch-GPI] suggests a low level of cross-protection, which was reflected in oocyst output (17% to 27% reduction; Fig. 5B and Supplementary Fig. S3). We also observed that increased serum IL-10 levels may lead to increased oocyst shedding, which agrees with previous findings (Sand et al., 2016), indicating that serum IL-10 levels could be a potential predictive marker of susceptibility to infection (Wu et al., 2016).

Previous studies have shown that fecundity of *Eimeria* parasites is reduced as the infective dose increases due to a 'crowding effect', and infection with 250 oocysts is sufficient to induce the highest level of oocyst shedding (Johnston et al., 2001; Williams, 2001). For this reason, measurement of oocyst replication after a low-dose challenge is the most sensitive method available for measuring vaccine efficacy, and is still regarded as a 'gold standard' by vaccine regulators. However, this approach is not suitable for evaluation of factors such as protection against intestinal damage (lesion score) and body weight gain as shown by a recent study, where infections with similar doses of *E. maxima* only induced mild lesions and very low body weight losses (Jenkins et al., 2017a). Future trials will be required to evaluate protection against more severe challenge doses.

The confirmation of limited genetic diversity between AMA1 alleles for *E. maxima*, with no evidence of significant signatures of selection, mirrors that described previously for *E. tenella* (Blake et al., 2015). Antigenic diversity within parasite populations provides the potential for immune escape, with the greatest likelihood when small numbers of antigens are used in subunit or recombinant vaccines to induce focused immune responses. The low levels of naturally occurring diversity described for *EtAMA1* have enhanced its candidacy for use in novel anti-coccidial vaccines. While AMA1 is known to be highly polymorphic within populations of some apicomplexans such as *Plasmodium falciparum* (Takala and Plowe, 2009), the low levels of polymorphism and its ability to induce immune protection described for two *Eimeria* spp. suggest that AMA1 is a viable vaccine candidate for all *Eimeria* spp. which infect chickens, and possibly all *Eimeria* spp.

Currently the commercial availability of live vaccine formulations is constrained by inherent limitations in the capacity of production of live parasites, which is especially true where attenuated vaccines are produced (Williams, 1998). In the broiler sector, where profit margins are very tight, control measures are still highly dependent on the use of anti-coccidial prophylaxis due to the higher costs of these vaccines (Sharman et al., 2010; Blake and Tomley, 2014). Therefore, there is a need to reduce the cost of the available formulations in the market and make them more attractive for this sector. Herein we show that *EtAMA1* is an effective vaccine candidate, provide evidence for the first time that genetic complementation of *E. tenella* parasites is a valuable tool to deliver vaccine candidates from other coccidian species which infect poultry, and demonstrate that this vaccine platform is able to induce significant levels of heterologous protection after complementation with *EmAMA1*. Furthermore, we provide a powerful tool to develop new generation vaccines against multiple *Eimeria* spp. as a means to streamline the available formulations in the market, and thus reduce their cost. In this sense, successful genetic complementation of highly prolific and less pathogenic species such as *Eimeria acervulina* would be highly beneficial (Zou et al., 2009).

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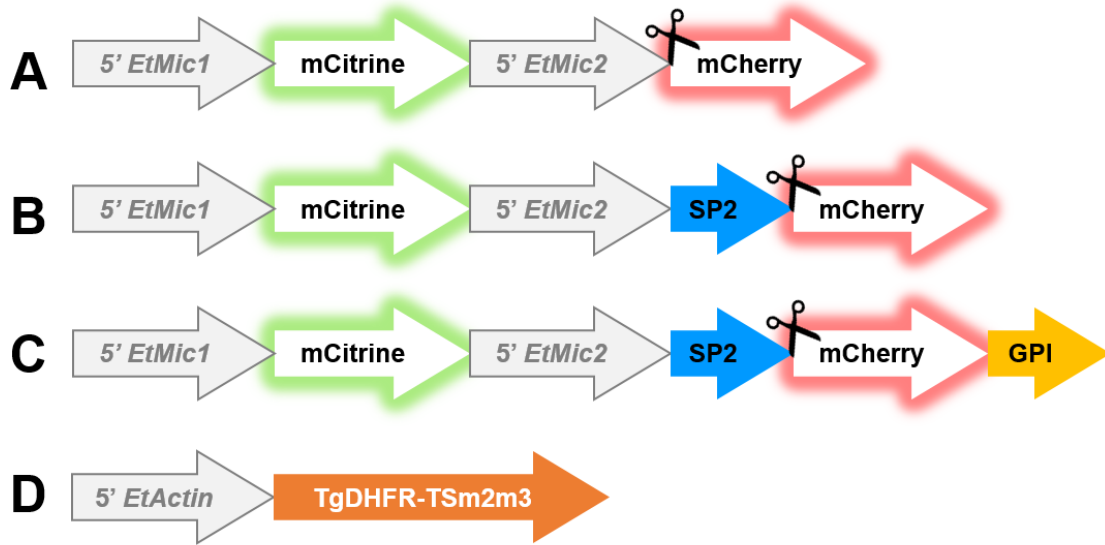
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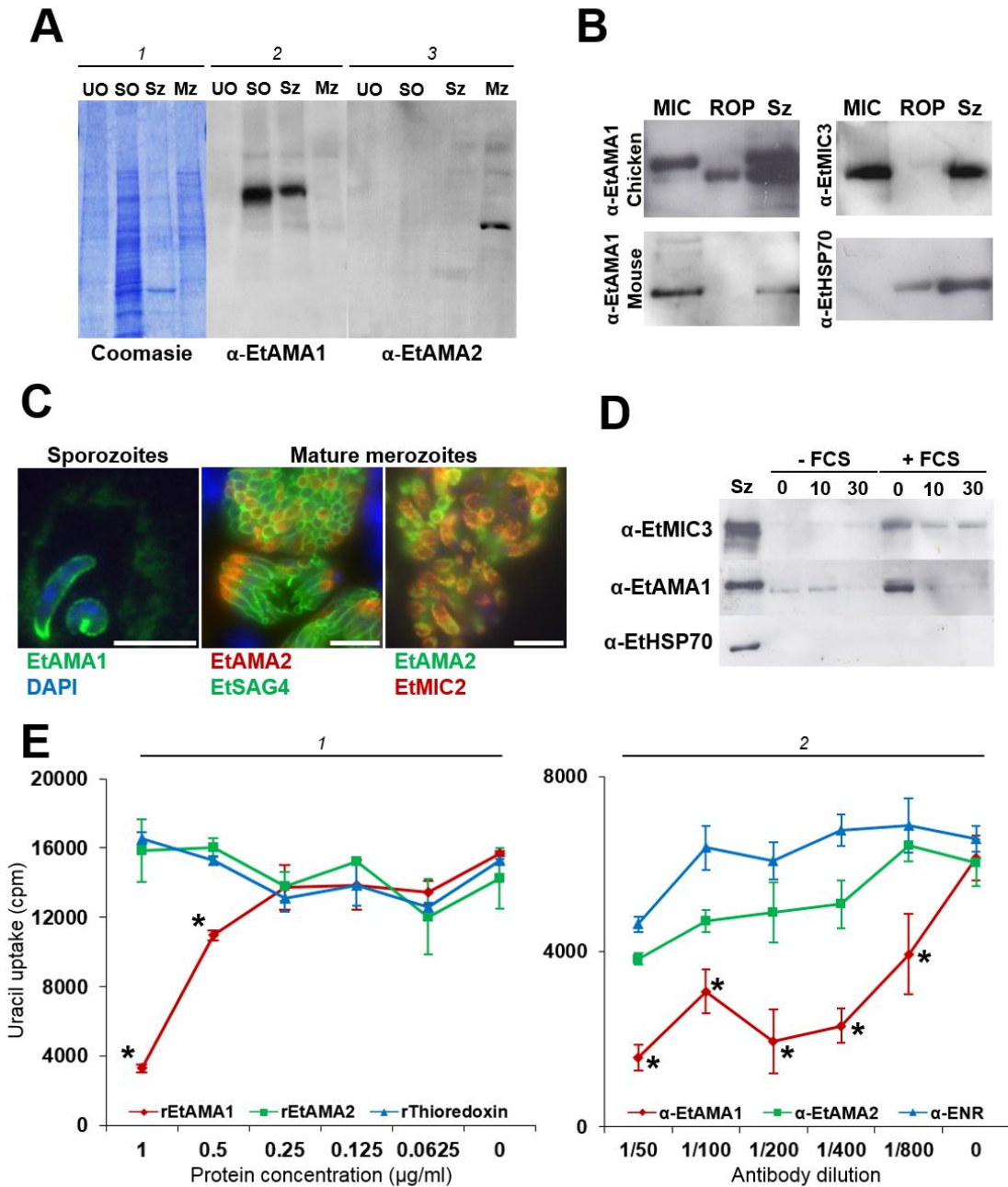
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Figure legends

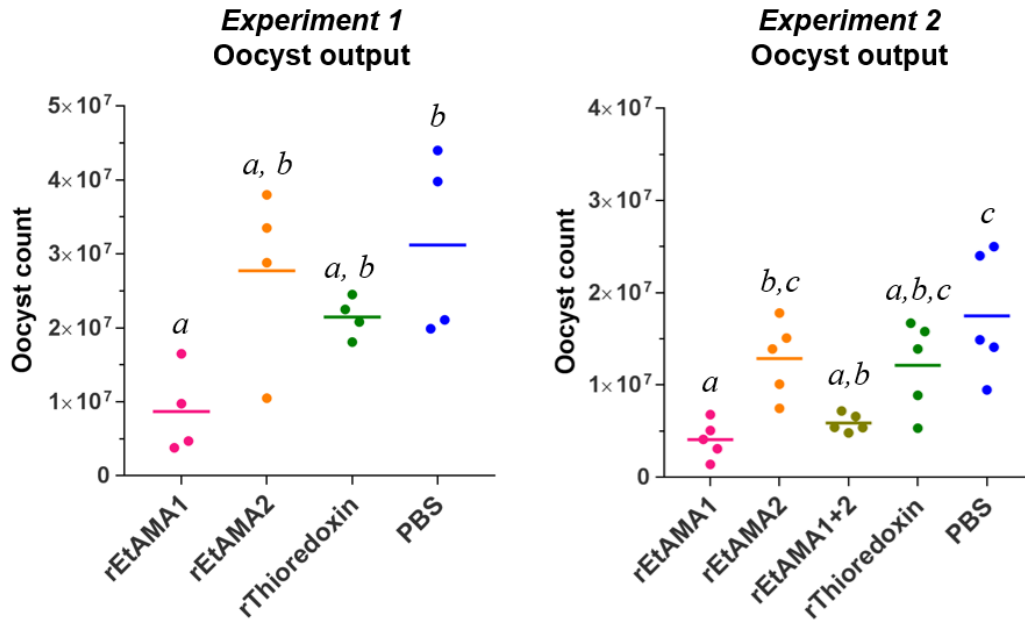


**Fig. 1.** Simplified representation of the plasmids used for *Eimeria tenella* transfection. (A-C) Core constructs employed to transfect *E. tenella* parasites. Scissors represent the location of the unique *XbaI* restriction site, used for transgene insertion. In transfected parasites, the mCitrine protein is expressed as a cytosolic protein and used to select transgenic parasites by flow cytometry, whereas mCherry, or transgene-mCherry fusion protein, is expressed as a cytosolic protein (with no delivery signals), secreted into the sporocyst cavity (SP2), or secreted and anchored onto the sporozoite surface (SP2+GPI) (Clark et al., 2012; Marugan-Hernandez et al., 2017). (D) A plasmid carrying the mutant *Toxoplasma gondii* dihydrofolate reductase–thymidylate synthase (DHFR-TSm2m3) gene was used for in vivo selection of transgenic parasites resistant to pyrimethamine treatment (Clark et al., 2008).

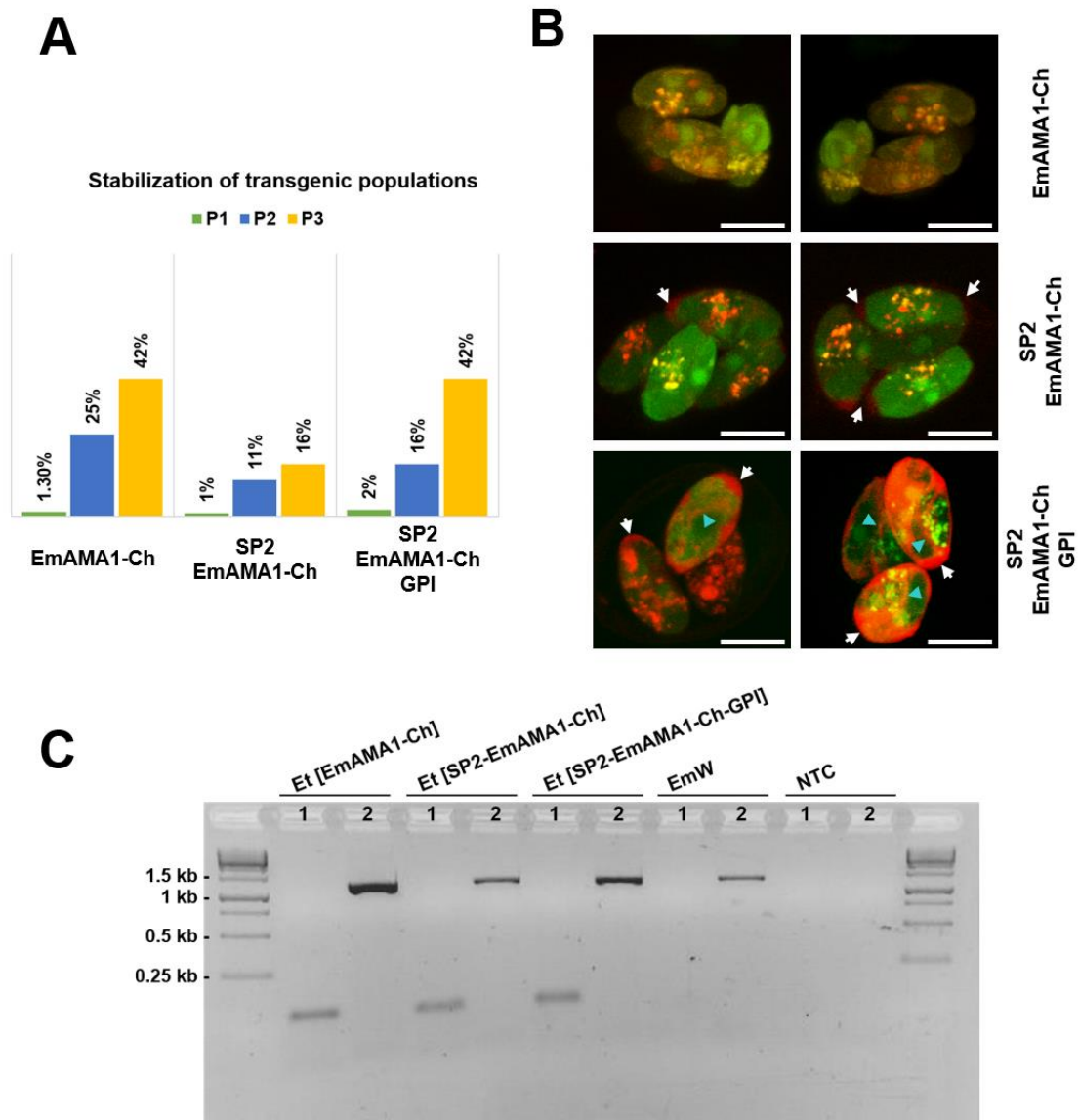


**Fig. 2.** Characterisation of the *Eimeria tenella* apical membrane antigens (EtAMA) 1 and 2. (A) Protein extracts obtained from unsporulated oocysts (UO), sporulated oocysts (SO), sporozoites (Sz) and merozoites (Mz) resolved by SDS-PAGE and stained using Coomassie brilliant blue R-250 (1). Detection of EtAMA1 (2) and EtAMA2 (3) on the same protein extracts by western blotting using specific hyperimmune mouse sera. EtAMA1 was restricted to sporulated oocysts and sporozoites, whereas EtAMA2 was only found in merozoites. (B) Detection of EtAMA1 on micronemes (MIC), rhoptries (ROP)

and sporozoites (Sz) by western blotting. Mouse antiserum recognised a single microneme protein (~63 kDa). Antibodies raised against the *E. tenella* microneme protein 3 (*EtMIC3*) and the *E. tenella* heat shock protein 70 (*EtHSP70*) confirmed that microneme fractions were not contaminated with rhoptries, and vice versa. (C) Detection of *EtAMA1* on sporozoites and *EtAMA2* on mature second generation merozoites by IFAT. Some *EtAMA2* sections were counterstained with an antibody against the *E. tenella* surface antigen 4 (*EtSAG4*) to delineate the sporozoites, and some others with an antibody against the *E. tenella* microneme protein 2 (*EtMIC2*) to delineate the micronemes. *EtAMA1* and *EtAMA2* showed apical localisations within the zoites. *EtAMA2* partially co-localised with *EtMIC2* in merozoites. Bars represents 10  $\mu$ m. (D) Detection of *EtAMA1* on sporozoites (Sz) and sporozoite secreted fractions (0-30) by western blotting with or without treatment with FCS (-/+ FCS) and collected at 0, 10 or 30 min post-incubation. A low level of *EtAMA1* secretion was constitutive, but secretion was strongly induced after FCS supplementation. *EtMIC3* detection was used as positive control of secretion, while *EtHSP70* detection was used to show the absence of sporozoite lysis. (E) In vitro sporozoite inhibition assays using recombinant (r) *EtAMA1* and *EtAMA2* (1) or specific anti-sera (2). All the experiments were done in triplicate. The recombinant thioredoxin protein (rThioredoxin) and a mouse antibody against parasite enolyl reductase (ENR) were included as control treatments for each respective protocol. Only treatment with r*EtAMA1* or anti-*EtAMA1* serum induced a significant reduction in parasite replication. \* indicates statistical differences ( $P<0.05$ ).



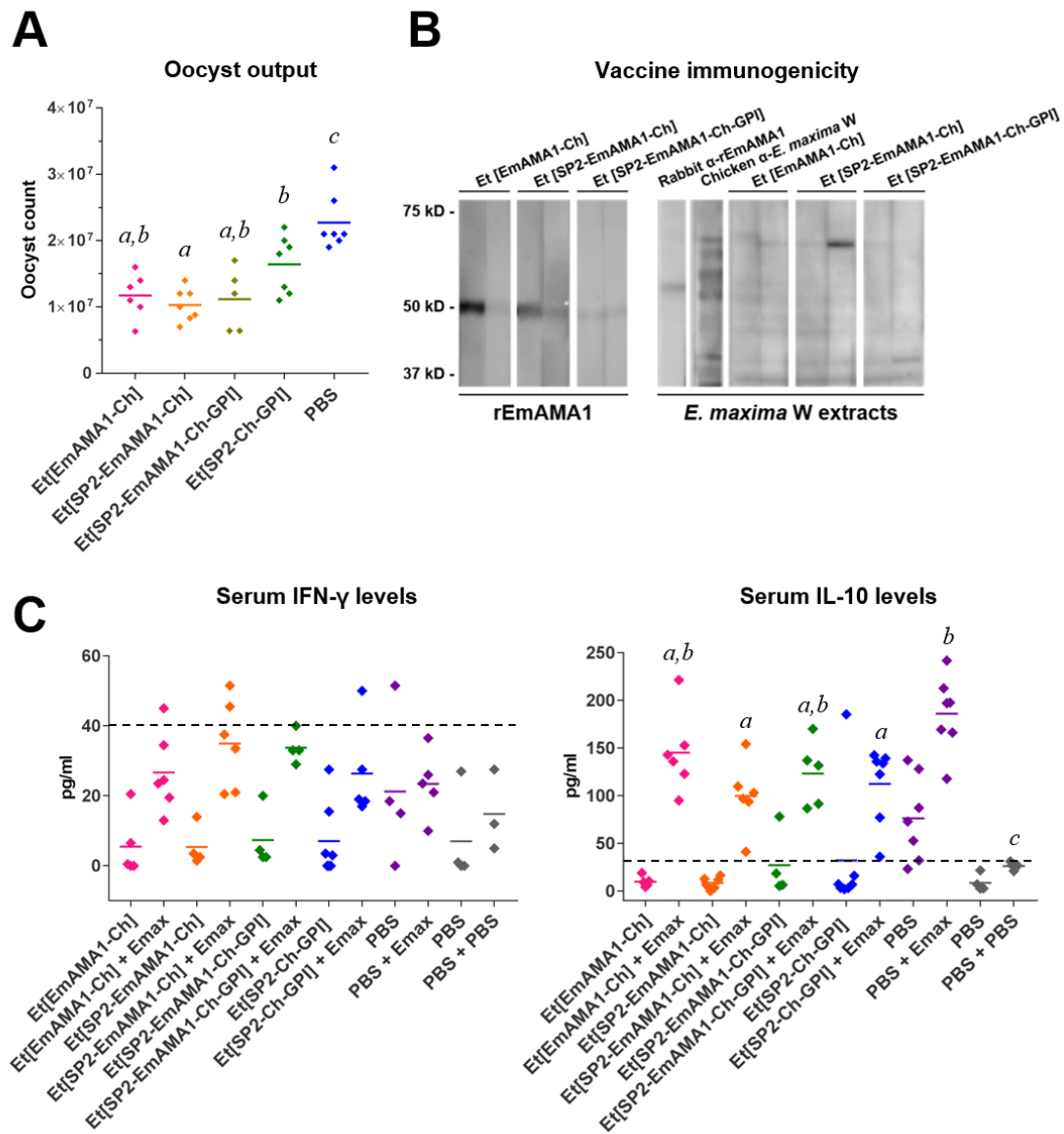
**Fig. 3.** Immunising ability of recombinant (r) *Eimeria tenella* apical membrane antigen (EtAMA) 1, rEtAMA2 and their combination against challenge with *E. tenella* H parasites (Experiment 1,  $n=4$ ; Experiment 2,  $n=5$ ). In each experiment a group of birds was vaccinated with the Trx-tag present in the recombinant proteins (rThioredoxin), and an additional group was mock vaccinated using PBS. Dots illustrate individual oocyst counts and bars indicate average values. Only birds vaccinated with rEtAMA1, alone or in combination, displayed a significant reduction in oocyst shedding. Groups marked with different letters were significantly different ( $P<0.05$ ).



**Fig. 4.** Characterisation of the three different populations of *Eimeria tenella* expressing the *Eimeria maxima* apical membrane antigen 1 (*EmAMA1*) fused to mCherry (Ch). (A) Stabilization of transgenic populations by successive in vivo passages. Percentages indicate the total proportions of transgenic oocysts passage by passage (P1 to P3). (B) Fluorescent patterns observed by confocal microscopy in stable transgenic populations of *E. tenella* oocysts expressing the *EmAMA1*-Ch protein. mCitrine was always observed in the cytosol for all the populations, whereas mCherry was observed in the cytosol (*EmAMA1*-Ch, with no delivery signals), secreted into the sporocyst cavity (*SP2-EmAMA1*-Ch, white arrows), or secreted into the sporocyst cavity (white arrows) and anchored onto the sporozoite surface (*SP2-EmAMA1*-Ch-GPI, white and blue arrows respectively). In all the populations both

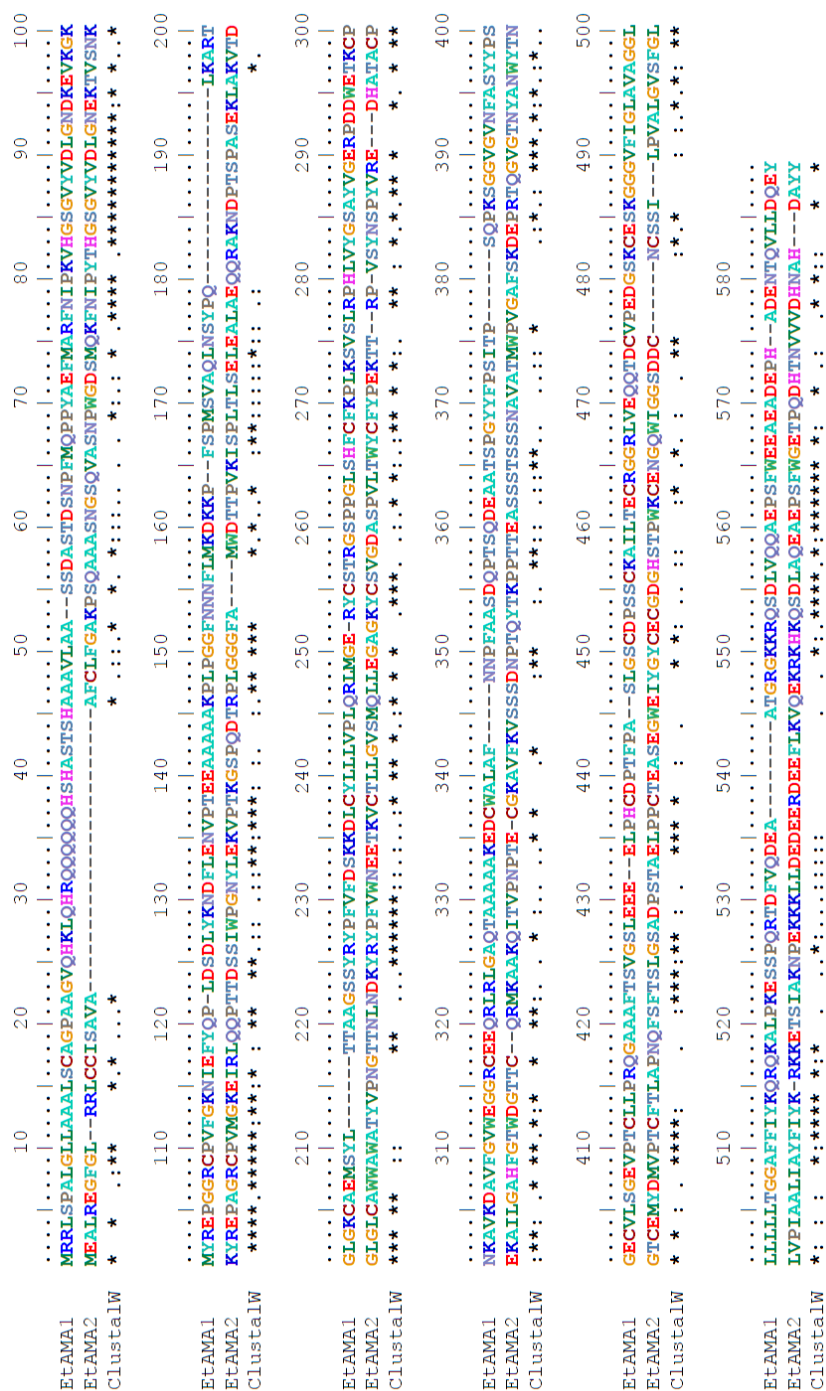
mCitrine and mCherry were also present within cytosolic aggregates and in some cases were co-localised. Bars represent 10  $\mu$ m. (C) Detection of *EmAMA1*-Ch transcripts in cDNA isolated from stable transgenic populations by reverse transcription (RT)-PCR. Presence of genomic DNA contamination was discarded by the use of *EtActin* primers that amplify a region coded between two adjacent exons (~0.1 kb, lane 1). A single band of ~1.3 kb was obtained from all three populations when specific primers targeting *EmAMA1* were employed (lane 2). cDNA isolated from *Eimeria maxima* W strain (EmW) was included as a positive control reaction. NTC is the non-template control.





**Fig. 5.** Immunogenicity and efficacy of *Eimeria tenella* parasites expressing the *Eimeria maxima* apical membrane antigen 1 (*EmAMA1*) under the control of different delivery signals. (A) Immunising ability of *Eimeria tenella* Wis strain parasites expressing *EmAMA1* protein fused to mCherry (Ch) and expressed under the control of different delivery signals against challenge with *E. maxima* W strain parasites. SP2, signal peptide from the *E. tenella* microneme 2 protein (*EtMIC2*); GPI, glycosylphosphatidylinositol -anchor from the *E. tenella* surface antigen 1 (*EtSAG1*). A group of birds was vaccinated with an empty vaccine vector (the most complete, carrying the SP2 and GPI signals), and an additional group was not vaccinated (PBS). Diamonds illustrate individual oocyst counts and

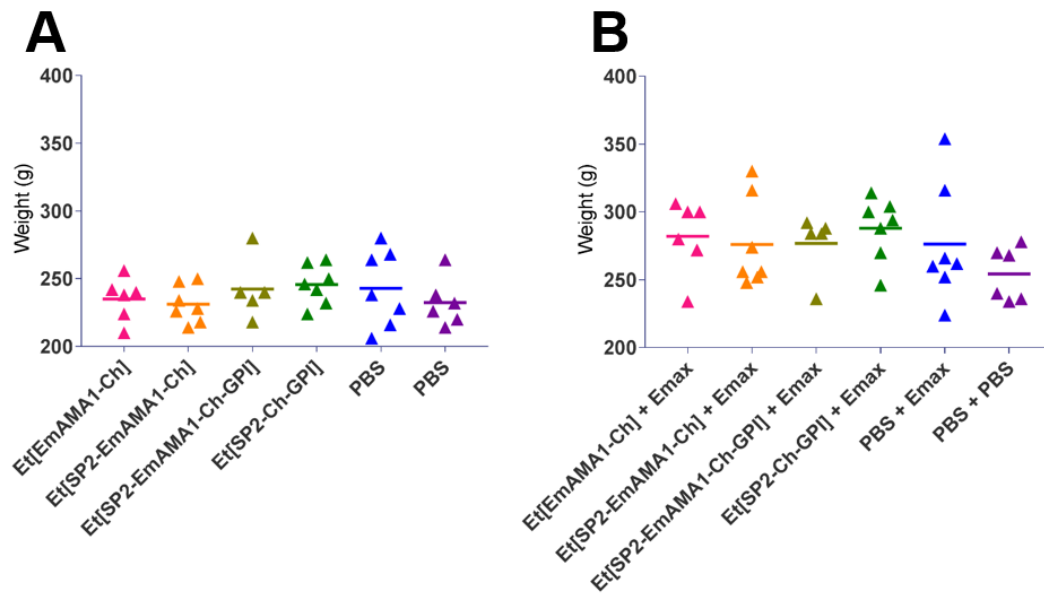
bars indicate average values. All the birds vaccinated with *E. tenella* parasites expressing *EmAMA1*-Ch displayed a significant reduction in oocyst shedding, regardless of the delivery signal included. Groups marked with different letters were significantly different ( $P < 0.05$ , one-way ANOVA test). (B) IgY responses induced after vaccination with *Et[EmAMA1-Ch]*, *Et[SP2-EmAMA1-Ch]*, and *Et[SP2-EmAMA1-Ch-GPI]* by western-blot. Recombinant *EmAMA1* (*rEmAMA1*) and *E. maxima* W protein extracts were separated by SDS–PAGE and probed with sera collected from vaccinated birds. Some birds strongly recognized the recombinant protein, but high variability within groups was observed. In contrast, none of the birds recognized native *EmAMA1* from oocyst extracts, which could be detected with rabbit anti-*rEmAMA1*. (C) Quantification of IFN- $\gamma$  and IL-10 serum levels on day 29, (8 days after the final vaccination with transgenic parasites) or at day 36 (6 days after challenge with *E. maxima* W, marked with '+ Emax' on the axis labels). Diamonds illustrate individual serum concentrations and bars indicate average values. Letters on the right plot indicate significant differences between groups after the challenge infection ( $P < 0.05$ , one-way ANOVA test). Dotted lines depict threshold values for IFN- $\gamma$  (40 pg/ml) and IL-10 (32 pg/ml) tests.



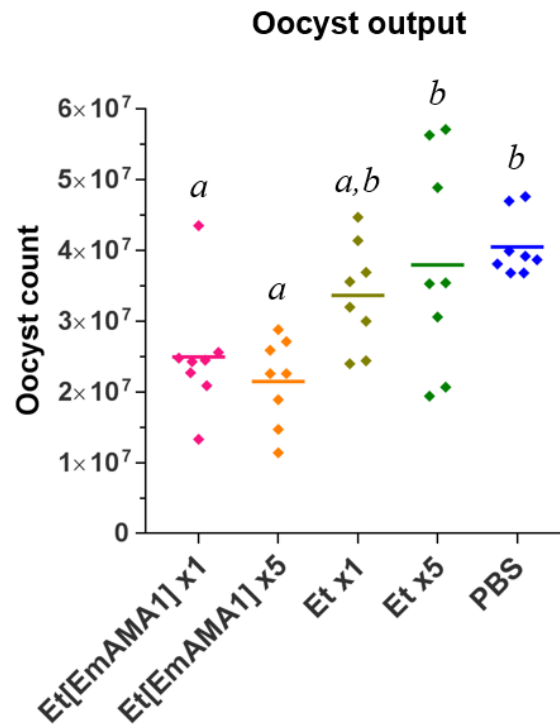
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986 **Supplementary Fig. S1.** Sequence alignment of the *Eimeria tenella* apical membrane antigens 1  
 987 (*EtAMA1*, ToxoDB Accession number ETH\_00007745) and 2 (*EtAMA2*, ToxoDB Accession number  
 988 ETH\_00004860). Asterisks (\*) indicate fully conserved residues. Colons (:) and periods (·) indicate  
 989 conservation between groups of strongly or weakly similar properties, respectively.

990



**Supplementary Fig. S2.** Safety of transgenic vaccines based on genetically complemented *Eimeria tenella* parasites expressing the *Eimeria maxima* apical membrane antigen 1 (*EmAMA1*). fused to mCherry (Ch) and under the control of different delivery signals (SP2, signal peptide from the *E. tenella* microneme 2 protein (*EtMIC2*); GPI, glycosylphosphatidylinositol -anchor from the *E. tenella* surface antigen 1 (*EtSAG1*)). Individual weights from birds vaccinated with transgenic parasites expressing *EmAMA1* (*Et[EmAMA1-Ch]*, *Et[SP2-EmAMA1-Ch]*, *Et[SP2-EmAMA1-Ch-GPI]*), birds vaccinated with the empty vector (*Et[SP2-Ch-GPI]*), and non-vaccinated birds (PBS) 7 days after the final vaccination (A) or 6 days after challenge infection ('+ Emax', B). Triangles illustrate individual weights and bars indicate average values. No differences were found between groups ( $P>0.05$ ).



**Supplementary Fig. S3.** Immunising ability of *Eimeria tenella* Wis strain parasites expressing the *Eimeria maxima* apical membrane antigen 1 (*EmAMA1*) under the control of the *E. tenella* AMA1 promoter (*Et[EmAMA1]*). A total of 40 3 weeks old Light Sussex chickens were split into five groups of eight in independent wire-floored cages and housed within, coccidia-free conditions. Birds were dosed by oral gavage with *Et[EmAMA1]* oocysts one (x1) or five times (x5), or with wild type *E. tenella* Wis strain (*Et*) oocysts one (x1) or five times (x5), or with PBS. Three weeks after the last vaccination, birds from all groups were challenged with 300 oocysts of the *E. maxima* W strain, and individual faecal samples were collected from 5 to 9 days post-challenge to determine the total oocyst output. Dots illustrate individual oocyst counts and bars indicate average values. All the birds vaccinated with *Et[EmAMA1]* parasites displayed a significant reduction in oocyst shedding, regardless of the number of doses received. Groups marked with different letters were significantly different ( $P < 0.05$ ).

**Table 1.** Experimental design for the vaccine trials using recombinant (r) *Eimeria tenella* apical membrane antigens 1 (*EtAMA1*) and 2 (*EtAMA2*).

Experiment	<i>n</i>	Vaccine	Immunisation protocol	Age at <i>EtH</i> challenge
1	4	PBS	500 µl at 1, 3 and 5 wks	7 wks
	4	rThioredoxin (protein tag)	100 µg at 1, 3 and 5 wks	7 wks
	4	r <i>EtAMA1</i>	100 µg at 1, 3 and 5 wks	7 wks
	4	r <i>EtAMA2</i>	100 µg at 1, 3 and 5 wks	7 wks
2	5	PBS	500 µl at 1, 3 and 5 wks	7 wks
	5	rThioredoxin (protein tag)	100 µg at 1, 3 and 5 wks	7 wks
	5	r <i>EtAMA1</i>	100 µg at 1, 3 and 5 wks	7 wks
	5	r <i>EtAMA2</i>	100 µg at 1, 3 and 5 wks	7 wks
	5	r <i>EtAMA1</i> + r <i>EtAMA2</i>	100 µg at 1, 3 and 5 wks	7 wks

Wks, weeks; *EtH*, *Eimeria tenella* Houghton strain.

**Table 2.** Experimental design for the vaccine trials using *Eimeria tenella* parasites (*Et*) expressing the *Eimeria maxima* apical membrane antigen 1 (*EmAMA1*). fused to mCherry (Ch) and under the control of different delivery signals (SP2, signal peptide from the *E. tenella* microneme 2 protein (*EtMIC2*); GPI, glycosylphosphatidylinositol -anchor from the *E. tenella* surface antigen 1 (*EtSAG1*)).

Group	<i>n</i>	Vaccine	Immunisation protocol	Age at <i>EmW</i> challenge (days)
1	7	<i>Et[EmAMA1-Ch]</i>	100 oocysts at day 3	30
2	7	<i>Et[SP2-EmAMA1-Ch]</i>	500 oocysts at day 9	30
3	7	<i>Et[SP2-EmAMA1-Ch-GPI]</i>	3,000 oocysts at day 15	30
4	7	<i>Et[SP2-GPI]</i> , empty vector	5,000 oocysts at day 22	30
5	7	PBS	PBS at days	30
6	7	PBS	3, 9, 15 and 22	No challenge

*EmW*, *Eimeria maxima* Weybridge strain.

**Table 3.** *Eimeria* apical membrane antigen (AMA1-4) gene identities (ID), transcription profiles and evidence of selective pressure (mean Ka/Ks ratios for six *Eimeria* spp. which infect chickens compared to *Eimeria tenella*). *Eimeria tenella* gene-specific transcription profiles are presented as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) normalised against average FPKM for the full relevant dataset.

	<i>E. maxima</i> gene ID	<i>E. tenella</i> gene ID	<i>E. tenella</i> transcription profiles (FPKM)								Selective pressure? Mean Ka/Ks <sup>c</sup>
			UO_1 <sup>c</sup>	UO_2 <sup>c</sup>	SO <sup>c</sup>	Sz <sup>c</sup>	Sz <sup>d</sup>	Mz2 <sup>c</sup>	Mz2 <sup>d</sup>	Gam <sup>d</sup>	
AMA1	FN813221 <sup>a</sup>	ETH_00007745 <sup>b</sup>	0.010	0.006	6.89	25.47	33.67	0.028	0.169	0.057	0.12
AMA2	EMWEY_00006480 <sup>b</sup>	ETH_00004860 <sup>b</sup>	0.000	0.000	0.041	0.015	0.029	4.21	0.510	4.612	na
AMA3	?	ETH_00017730 <sup>b</sup>	0.035	0.031	2.64	4.16	16.05	0.005	0.026	0.074	na
AMA4	EMWEY_00022320 <sup>b</sup>	ETH_00013620 <sup>b</sup>	0.001	0.002	2.58	2.39	2.60	1.97	2.08	3.04	0.11

UO, unsporulated oocyst; SO, sporulated oocyst; Sz, sporozoite; Mz2, second generation merozoite; Gam, gametocyte.

<sup>a</sup>GenBank Accession Number.

<sup>b</sup>ToxoDB Accession Number.

<sup>c</sup>Data derived from Reid et al. (2014).

<sup>d</sup>Data derived from Walker et al. (2015).

**Table 4.** Summary of genetic diversity for the *Eimeria maxima* and *Eimeria tenella* AMA1 orthologous coding sequences. Data for *E. tenella* are reproduced from Blake et al. (2015).

	<i>E. maxima</i>	<i>E. tenella</i>
<i>n</i>	18	56
Continents sampled	3	5
S	10 (9)	13 (13)
dN	3	10
dS	7	3
K	3.49	4.04
$\pi$ Jukes Cantor	0.0028	0.0032
H	9	8
Hd	0.922	0.771
Tajima's D	0.722 (ns)	1.259 (ns)
Fu and Li's D*	0.953 (ns)	1.513 (0.05)
Fu and Li's F*	1.026 (ns)	1.688 (0.05)

*n*, the number of sequences tested; S, the number of variant sites detected, with the number of parsimony-informative variant sites shown in parentheses; dN, the number of non-synonymous variant sites; dS, the number of synonymous variant sites; k, the average number of pairwise differences;  $\pi$ , nucleotide diversity, calculated with the Jukes Cantor correction; H, the number of sequence haplotypes detected; Hd, the haplotype diversity; ns, not significant.

Tajima's D and Fu and Li's D\* and F\* tests were used to assess the extent or neutrality of signatures of selection with significance (*P*) shown in parentheses where relevant.